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(54) **Insect-resistant tomato plants.**

(57) A method for producing genetically transformed tomato plants exhibiting toxicity to Lepidopteran larvae is disclosed. In another aspect, the present invention embraces chimeric plant genes, genetically transformed tomato cells and differentiated tomato plants which exhibit toxicity to Lepidopteran larvae.

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Description

INSECT-RESISTANT TOMATO PLANTS

Bacillus thuringiensis is a spore forming solid bacterium which is known for its ability to produce a parasporal crystal protein which is toxic to a wide variety of Lepidopteran larvae. The crystals, which account for about 20-30% of the dry weight of sporulated cultures, consist primarily of a single, high molecular weight protein (~134 kilodaltons (KD)) which is produced only during sporulation. The crystal protein is produced in the bacterium as the protoxin which is activated in the gut of susceptible larvae to produce a toxic protein having a molecular weight of about 67 KD.

Insects susceptible to the action of the protein toxin of Lepidopteran-type *Bacillus thuringiensis* bacteria include, but are not limited to, tomato pinworm (*Keiferia lycopersicella*), tobacco hornworm (*Manduca sexta*), tomato hornworm (*Manduca quinquemaculata*), beet armyworm (*Spodotera exigua*), cabbage looper (*Trichoplusia ni*), and tomato fruitworm (*Heliothis zea and Heliothis virescens*). Larvae of these insects are known to feed on tomato plants.

Commercial insecticidal preparations containing spores and crystalline protein produced by *Bacillus thuringiensis* are available under such names as DIPEL® and THURICIDE®. Significant limitations in the use of commercial preparations of the crystalline protein toxin of *B. thuringiensis* include the need for repeated applications of the insecticidal preparations. Another disadvantage is that the crystal protein is only produced during the sporulation stage of the life cycle of *B. thuringiensis.* Such a growth phase limitation, particularly in an industrial process, can result in inconvenience and excessive time requirements during manufacture. At the completion of sporu lation, the self-lysing cells release both spores and crystals into the culture medium. Because of environmental concerns it is desirable that commercial insecticidal preparations be substantially free of spores. Unfortunately, because of the similarity in size and density of the spores and crystal protein toxin, separation of the crystals from the spores is complicated and laborious and thus, costly. Further, pressures resulting from growth phase limitations or other factors may result in preparations of *B. thuringiensis* losing their ability to produce the crystals; such acrystalliferous strains do not have insecticidal activity.

Although the isolation of DNA from *B. thuringiensis* coding for the crystal protein toxin and the insertion of this DNA into expression vectors for the transformation of bacterial species is known, the prior art does not teach with particularity and enablement how such DNA can be inserted into the genome of a tomato plant such that the toxin will exhibit insecticidal activity against susceptible Lepidopteran larvae.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the coding sequence for a crystal protein toxin of *Bacillus thuringiensis* subspecies *kurstaki* HD-1.
Figure 2 illustrates the preparation of plasmid pMON9733.
Figure 3 illustrates the preparation of plasmid pMAP17.
Figure 4 illustrates the preparation of plasmid pMON294.
Figure 5 illustrates the preparation of plasmid pMON8053.
Figure 6 illustrates the preparation of plasmid pMON9712.
Figure 7 illustrates the preparation of plasmid pMON9711.
Figure 8 illustrates the preparation of plasmid pMON9713.
Figure 9 illustrates the preparation of plasmid pMON9741.
Figure 10 illustrates the preparation of plasmids pMON9755, pMON9756 and pMON9757.
Figure 11 illustrates the make-up of plasmid pMON316.

STATEMENT OF THE INVENTION

The present invention provides a method for transforming tomato plants to be toxic to susceptible Lepidopteran larvae. More particularly, the present invention provides transgenic tomato plants which express the toxin protein of *Bacillus thuringiensis* at an insecticidal level.

In one aspect, the present invention comprises chimeric genes which function in tomato plants and produce transgenic tomato plants which exhibit toxicity toward susceptible Lepidopteran larvae. The expression of a plant gene which exists as double-stranded DNA form involves the transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase, and processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the mRNA.

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter". The promoter region contains a sequence of nucleotides which signals RNA polymerase to associate with the DNA, and initiate the production of a mRNA transcript using the DNA strand downstream from the promoter as a template to make a corresponding strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS), octopine synthase (OCS) and mannopine synthase (MAS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the cauliflower mosaic virus (CaMV) 19S and 35S promoters, and the light-inducible promoter from the small subunit of ribulose bis-phosphate

carboxylase (ssRUBISCO, a very abundant plant polypeptide). These types of promoters have been used to create various types of DNA constructs which have been expressed in plants; see e.g., PCT publication WO 84/02913 (Rogers et al, Monsanto).

Promoters which are known or are found to cause production of a mRNA transcript in plant cells can be used in the present invention. The promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of toxin protein to render the plant toxic to Lepidopteran larvae. Those skilled in the art recognize that the amount of toxin protein needed to induce the desired toxicity may vary with the particular Lepidopteran larvae to be protected against. Accordingly, while the CaMV35S and MAS promoters are preferred, it should be understood that these promoters may not be optimal promoters for all embodiments of the present invention.

The mRNA produced by the chimeric gene also contains a 5′ non-translated leader sequence. This sequence may be derived from the particular promoter selected such as the CaMV35S or MAS promoters. The 5′ non-translated region may also be obtained from other suitable eukaryotic genes or a synthetic gene sequence. Those skilled in the art recognize that the requisite functionality of the 5′ non-translated leader sequence is the enhancement of the binding of the mRNA transcript to the ribosomes of the plant cell to enhance translation of the mRNA in production of the encoded polypeptide.

The chimeric gene also contains a structural coding sequence which encodes the toxic protein of *Bacillus thuringiensis* or an insecticidally-active fragment thereof. It is known that Lepidopteran-type *B. thuringiensis* bacteria typically contains three toxin genes which have been classified on the basis of specific HindIII restriction fragments associated with each gene, see Kronstad et al. (1983). These genes are usually referred to as 4.5, 5.3 and 6.6 genes based on the size of the HindIII fragments obtained therefrom. It has been further shown that fragments of the complete toxin are insecticidally-active toward Lepidopteran larvae. Hence, for purposes of the present invention it should be understood that one can derive the coding sequence for an insecticidally-active toxin from any of the three genes and may further derive insecticidally-active fragments thereof without employing undue experimentation. Hence, for purposes of the present invention by "toxin protein" is meant either the full-length toxin as naturally produced by *Bacillus thuringiensis* or fragments thereof ("truncated toxin") possessing insecticidal activity toward the aforementioned Lepidopteran larvae. It has been found that truncated toxin coding sequences are more easily expressed and, therefore, are preferred. Furthermore, introduction of superfluous DNA, not required for the toxin acitivity, into the genome of the plant is also reduced.

Those skilled in the art will recognize that promoters useful in a particular embodiment will necessarily depend on the stability of the mRNA transcript. Indeed, as noted above, the truncated toxin coding sequences are preferred since they appear to produce more stable mRNA transcripts which are more easily expressed. Accordingly, promoters that are not effective or less effective with full-length toxin coding sequences can often be used with truncated toxin coding sequences to produce an effective amount of toxin protein.

For clarity and brevity of explanation, the following examples will describe chimeric gene constructs employing structural coding sequences derived from a "5.3 type" toxin gene from from an isolate *B. thuringiensis* subspecies *kurstaki* HD-1. Those skilled in the art recognize that other subspecies of *Bacillus thuringiensis* exhibit toxicity toward the aforementioned Lepidopteran insects. These other subspecies include *B.t. kurstaki* HD-1 Dipel (PCT publication WO 86/01536), *B.t. sotto, B.t. berliner, B.t. thuringiensis, B.t. tolworthi, B.t. dendrolimus, B.t. alesti, B.t. galleriae, B.t. aizawai and B.t. subtoxicus.* It should be understood that one may synthesize or isolate a coding sequence encoding a toxin protein from one of the above-identified *B.t* subspecies or others without undue experimentation and transform tomato plants to be toxic to susceptible Lepidopteran larvae as described herein. Accordingly, such variations and derivations are considered to be within the scope of the present invention. It should be further understood that the expression level may vary with the particular toxin coding sequence used and that the scope of toxicity toward Lepidopteran larvae may vary with the source of the toxin coding sequence (i.e. different *B.t.* subspecies).

Isolation of DNA sequences encoding the toxin protein of *B. thuringiensis* is well known in the art. The coding sequence from the above-identified *B.t.* subspecies are quite homologous, particularly in the N-terminus region of the coding sequence. This homology is useful in the isolation of toxin protein coding sequences, since a DNA probe useful in the isolation of *B.t.* subspecies *kurstaki* HD-1 as described hereinafter would be useful in the isolation of toxin coding sequences from other subspecies. The amino acid sequence of the crystal protein toxin gene isolated from *Bacillus thuringiensis* subspecies *kurstaki* HD-1 was partially determined according to the method of Hunkapiller et al (1983). These sequences were verified using the DNA sequence of the NH_2-terminal portion of the crystal protein gene disclosed by Wong et al (1983). Synthetic oligo-nucleotide sequences based on an amino acid sequence determined from the crystal protein polypeptide were prepared according to the procedure of Beaucage et al (1981). The oligonucleotide probes prepared are as shown in Table I below.

Table I

SYNTHETIC OLIGONUCLEOTIDE PROBES

Size	Probe Sequence	Area of B.t. Protein
14-mer	TGG GGA CCG GAT TC	1200 bp region
14-mer	GAA AGA ATA GAA AC	* 27-31 amino acid region
21-mer	CCT GAA GTA GAA-GTA TTA GGT	* 19-25 " " "

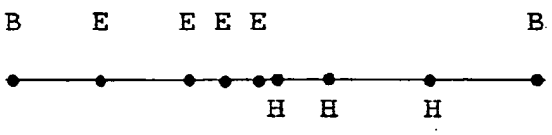
* numbered from NH_2- terminal end

Plasmid DNA from *B. thuringiensis* subspecies *kurstaki* HD-1 was purified from 1 to 2 liters of culture according to the procedure of Kronstad et al (1983). All plasmid preparations were banded at least once in CsCl/ethidium bromide gradients. Plasmids 30 megadaltons and larger in size were preferentially isolated.

Digestion with restriction enzymes EcoRI, PstI, HindIII, BamHI and SmaI, was carried out according to conditions recommended by the supplied (Boehringer Mannheim). *Escherichia coli* strain JM 101 (Messing et al. 1981) and strain SR-200 were used as the recipients for the transformation step. Competent cells were prepared according to standard procedures (Dagert et al. 1979). Colonies transformed with plasmid pUC8, were plated on L-agar with 100 µg/ml of ampicillin and 40 µl of 4% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (x-gal).

Plasmid DNA was transferred to nitrocellulose according to the procedure of Southern (1975). Prehybridization was done by incubating the nitrocellulose paper with the bound transferred DNA in prehyridization fluid, 10 × Denhardt's (0.2% BSA, 0.2% Ficoll, 0.2% polyvinylpyrrolidone) and 6 × SSC (0.9M NaCl, 0.09M sodium citrate) for 2-4 hours at 37°C. Hybridization was done by incubating the nitrocellulose paper for 8-10 hours with 10-11 ml of the prehybridization fluid and the labelled probe. After several washes with 6× SSC at increasing temperatures (30-45°C) the paper was exposed to X-ray film.

BamHI-restricted pBR328 (100ng), treated with alkaline phosphatase (Boehringer Mannheim) was mixed and ligated with 500 ng of *B. thuringiensis* plasmid DNA restricted with BamHI. $CaCl_2$ prepared competent E. coli SR200 were transformed and selected by ampicillin resistance and screened for tetracycline sensitivity. Analysis by mini-plasmid prep procedures (Maniatis et al. 1982) identified two clones which had the correct 16 Kb insert. Southern hybridization analysis with radiolabelled probes from Table I demonstrated that the DNA fragment which contained the sequence hybridizing to the synthetic probe had been sub-cloned. The two plasmids, designated pMAP1 and pMAP2, differed only in the orientation of the DNA fragment within the vector. These plasmid constructs produced material cross-reactive to *B.t.* crystal protein toxin antibody when analyzed according to Western blot procedures (Geshoni et al. 1979). A restriction map of the inserted *B.t.* fragment was prepared and four EcoRI (E) sites and three Hind III (H) sites were located between the BamHI (B) sites. This is schematically illustrated as:



E. coli strain SR200 containing pMAP2 has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA (hereinafter "ATCC") and has been designated ATCC accession number 39800.

An 8.1 Kb BamHI-PstI fragment was isolated after BamHI-PstI digestion of pMAP2 by electroelution from a preparative agarose gel onto DEAE paper used according to the directions of the manufacturer Schleicher & Schuell (see references). Plasmid pUC8 was used to sub-clone the BamHI-PstI fragment of pMAP2 carrying the *B.t.* gene. Ligation of pUC8 digested with BamHI and PstI with the purified 8.1 Kb BamHI- PstI fragment was followed by transformation of competent *E. coli* JM101. Transformants were selected on the basis of ampicillin

resistance and a lack of β-galactosidase activity. A clone was isolated and was confirmed to contain the desired plasmid. This construct was designated pMAP3. *E. coli strain* JM101 containing pMAP3 has been deposited with ATCC and has been designated ATCC accession number 39801.

Reduction of the *B. thuringiensis* DNA insert of pMAP3 from 8.1 Kb to 4.6 Kb was accomplished by deleting a SmaI-HpaI fragment. Plasmid pMAP3 DNA, purified by CsCl gradient centrifugation was digested with SmaI and HpaI restriction enzymes and religated. The resulting DNA fragment was utilized to transform competent *E. coli* JM101 cells. Ampicillin resistant transformants were screened by agarose electrophoresis of mini-plasmid preparations. A clone was identified which contained a plasmid with the expected DNA restriction enzyme digestion pattern. This construct was designated pMAP4.

Lastly, the chimeric gene contains a 3′ non-translated region which encodes a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3′ end of the mRNA. Examples of suitable 3′ regions are the 3′ transcribed, non-translated regions containing the polyadenylated signal of the nopaline synthase (NOS) gene of the *Agrobacterium* tumor-inducing (Ti) plasmid or the conglycinin (7S) storage protein gene. An example of a preferred 3′ non-translated region is that from the NOS gene, described in the following examples.

The chimeric gene of the present invention is inserted into the genome of a tomato plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens* as well as those described in e.g. Herrera-Estrella 1983, Bevan 1983, Klee 1985 and EPO publication 120,516, Schilperoort et al. In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *A. tumefaciens*, alternative methods can be used to insert the chimeric genes of this invention into tomato cells. Such methods may involve, for example, liposomes, electroporation and chemicals which increase free DNA uptake. Transformed cells are then cultured and regenerated into whole tomato plants.

The following examples are provided to better elucidate the practice of the present invention and are not intended, in any way, to limit the scope of the invention as claimed. Indeed, those skilled in the art will recognize that various equivalents, changes and modifications may be resorted to without departing from the spirit and scope thereof and it is understood that such equivalent embodiments are to be inclued herein.

EXAMPLE 1

The insecticidal toxin gene from *Bacillus thuringiensis* subspecies *kurstaki* HD-1 has been previously described by Watrud et al. (1985). The toxin gene is contained on a 4.6 kb fragment of *B.t.* DNA in plasmid pMAP4. The DNA sequence of 3734 nucleotides of pMAP4, including the entire toxin protein coding sequence, was determined by the chain termination method of Sanger et al. (1977). The DNA sequence and the derived amino acid sequence for the toxin protein are shown in Figure 1. Nucleotide coordinates referred to hereinafter are based on the numbering of Figure 1.

This sequence includes 171 nucleotides upstream of the translational initiation codon and extends through a KpnI site 188 nucleotides after the translational termination codon. The first nucleotide of the protein coding sequence is labeled position +1. DNA sequences from nucleotide -75 to nucleotide 220 and from nucleotide 3245 to 3650 were also determined by the chemical method of Maxam and Gilbert (1977). The DNA sequence from -171 to -160 is from the known sequence of the plasmid vector pUC7 (Vieira, 1982) DNA sequence from -159 to -153 is from a chemically synthesized PstI linker (New England Biolabs); the three nucleotides from -152 to -150 are derived from the known cleavage site for restriction enzyme HpaI. The sequence from nucleotide -149 to -76 has been inferred from known 5′-flanking sequences of other *B.t.* toxin genes (Schnepf et al. 1985, Thorne et al. 1986, Adang et al. 1985 and Shibano et al. 1985).

EXAMPLES 2-5

Chimeric *B.t.* Toxin Genes for Plant Transformation with CaMV35S Promoter

A. Full-Length Toxin

To make a chimeric gene encoding the toxin protein of *B.t.* a NcoI site is introduced at the translational initiation codon (ATG) of the DNA encoding the *B.t.* toxin such that the ATG codon is contained within the NcoI recognition site (CCATGG). DNA sequence analysis of the region of the toxin gene around the initiator codon revealed the sequence:

```
5'-GAGATGGAGGTAACTTATGGATAACAATCCGA-3'
        MetAspAsnAsnPro
```

To introduce the desired NcoI site, it was necessary to change the sequence around the ATG from TTATGG to CCATGG. Referring to Figure 2, a 340 bp DraI-EcoRI fragment which includes the translational initiation region was subcloned from pMAP4 between the SmaI and EcoRI sites of the filamentous bacteriophage vector M13mp8. This plasmid was named pMON9732. Single-stranded phage DNA from this construct contains the noncoding strand of the toxin gene sequence.

Site-specific mutagenesis was performed on single-stranded DNA from this construct by the method of Zoller and Smith (1983) utilizing as a primer a synthetic oligonucleotide of the sequence:

5'-GAGATGGAGGTAACCCATGGATAACAATCC-3'

Following mutagenesis a clone containing the desired change was identified by digestion with NcoI, and the presence of the NcoI site was confirmed by DNA sequence analysis. This clone was designated pMON9733.

An intact toxin gene was constructed which incorporated the NcoI site from the site-specific mutagenesis described above. Referring to Figure 3, pMAP3 was digested with BamHI and ClaI and a fragment of containing the pUC8 vector and the toxin gene from the ClaI site at position 1283 to the PstI site beyond the end of the gene was isolated. A 185 bp fragment extending from the BamHI site in the mp8 multilinker to the ClaI site at position 106 was isolated from pMON9733. These two fragments were ligated to create pMAP16. pMAP16 contains the NcoI site at the ATG but is missing the segment of the toxin gene between the ClaI sites at 106 and 1283. This ClaI fragment was isolated from pMAP4 and ligated with ClaI digested pMAP16. A plasmid containing this inserted ClaI fragment in the proper orientation to reconstruct a functional toxin gene was identified and designated pMAP17. *E. coli* containing this plasmid produced a protein of about 134,000 daltons which reacted with antibodies prepared against purified crystal toxin protein from *Bacillus thuringiensis* subspecies kurstaki HD-1 at levels comparable to those produced by *E. coli* containing pMAP4. *E. coli* containing pMAP17 were toxic to the Lepidopteran larvae *Manduca sexta*.

To facilitate construction of chimeric toxin genes in plant transformation vectors, BamHI and BglII sites were introduced just upstream of the NcoI site in the toxin gene. Referring to Figure 4, plasmid pMON146 was used as a source of a synthetic linker containing restriction sites for BamHI, BglII, XbaI and NcoI as shown:

```
5'-GGATCCAGATCTGTTGTAAGGAGTCTAGACCATGGATC-3'
   BamHI BglII          XbaI  NcoI
```

pMON146 was partially digested with PstI and then digested to completion with NcoI, and a 3.5 kb NcoI-PstI fragment was isolated. The 4.5 kb NcoI-PstI fragment containing the entire toxin gene was isolated from pMAP17, and this fragment was ligated with the 3.5 kb pMON146 fragment. A plasmid containing these two fragments was designated pMON294. In pMON294 a BamHI and a BglII site are just upstream of the initiation codon for the toxin protein, and a BamHI site is just downstream of the PstI site.

Referring to Figure 11, plasmid pMON316, a derivative of pMON200 (Fraley et al. 1985; Rogers et al, 1985) is a co-integrating type intermediate vector which contains the CaMV35S promoter and the 3' polyadenylation signal of the NOS gene. pMON316 has unique cleavage sites for the restriction endonucleases BglII, ClaI, KpnI, XhoI and EcoRI located between the 5' leader and the 3' NOS polyadenylation signals. The cleavage sites provide for the insertion of coding sequences carrying their own translational initiation signals immediately adjacent to the CaMV35S leader sequence. Plasmid pMON316 retains all the properties of pMON200 including spectinomycin resistance for selection in *E. coli and A. tumefaciens* as well as a chimeric kanamycin gene (NOS/NPTII/NOS) for selection of transformed plant tissue and the nopaline synthase gene for ready scoring of transformants and inheritance in progency.

Referring to Figure 5, a plasmid was constructed for the expression of the *B.t.* toxin gene in plants by ligating the 4.5 kb BamHI fragment containing the toxin gene from pMON294 into pMON316 which had been digested with BglII. A plasmid which contained the toxin gene oriented such that the translational initiator was adjacent to the CaMV35S promoter was identified by digestion with EcoRI and designated pMON8053. Another chimeric plant gene was prepared comprising the full-length construct in which the structural coding sequence for the *B.t.* toxin was truncated at the DraI site at position 3479. This site is 10 nucleotides beyond the translational terminator codon for the coding sequence for the full-length *B.t* toxin. Thus, this construct contains the full-length coding sequence but very little 3' flanking sequence from the *B.t.* subspecies *kurstaki* gene. This construct is designated pMON9712.

Referring to Figure 6, plasmid pMON9712 was prepared by digesting pMON294 with endonuclease DraI. A pair of complementary oligonucleotides having the following sequence were synthesized:

5'-TAGTAGGTAGCTAGCCA-3'
3'-ATCATCCATCGATCGGTCTAG-5'

When annealed to one another these oligonucleotides encode translational terminators in all three reading frames. The annealed oligonucleotide pair is flush-ended at one end and provides a four nucleotide single-stranded region capable of ligation to Bg1II digested DNA at the other end. The oligonucleotides were annealed to one other and ligated to pMON294 DNA which had been digested with DraI. The ligated DNA was digested with Bg1II, and a Bg1II fragment of approximately 3.5 kb containing the desired *B.t.* toxin coding sequence was isolated. This fragment extends from the Bg1II site just upstream of the translational initiation codon to the Bg1II site created by the oligonucleotide pair. This Bg1II fragment was ligated with Bg1II digested pMON316. A clone (pMON9712) was identified in which the translational initiator for the toxin gene was

adjacent to the 35S promoter by digestion with EcoRI.

B. Truncated Toxin

Previous work has shown that a fragment of the *B.t.* toxin coding sequence which extends from upstream of the translational initiator to the KpnI site at position 2170 produced a protein which is toxic to *M. sexta* when expressed in *E. coli*. A plant expression vector incorporating such a truncated *B.t.* toxin encoding gene was constructed as follows. Based on the DNA sequence of the toxin gene at the KpnI site at position 2170, a pair of complementary oligonucleotides was synthesized as shown below which when annealed to one another and ligated to the toxin gene at the KpnI site would encode two translational termination codons in frame with the toxin coding sequence.

5'-CTAGTAAA-3'
3'-CATGGATCATTTCTAG-5'

The annealed oligonucleotides provide, at one end, a four nucleotide single-stranded region capable of ligation to KpnI digested toxin gene, and, at the other end, a four nucleotide single-stranded reagion capable of ligation with BglII digested DNA. Referring to Figure 7, the olignucleotides were annealed to one another and ligated with pMON294 which had been digested with KpnI. This ligated DNA was then digested with BglII and a 2.2 kb BglII fragment was isolated. This fragment extends from the BglII site just upstream of the translational initiator of the toxin gene to the BglII site created by the oligonucleotide pair.

A plasmid was constructed for the expression in plants of the *B.t.* toxin gene truncated at the KpnI site by ligating the 2:2. kb BglII fragment with BglII digested pMON316. A clone was identified by digestion with EcoRI in which the translational initiator for the toxin gene was adjacent to the CaMV35S promoter and was designated pMON9711. Another chimeric plant gene was prepared comprising a truncated *B.t.* toxin of 646 amino acids corresponding to truncation at the Bc1I site at position 1935 of Figure 1. This gene construct was inserted into plant transformation vector pMON316 and designated pMON9713.

Briefly, pMON9713 was prepared as follows. Referring to Figure 8, plasmid pMON294 was digested with Bc1I. After digestion with BC1I the single-stranded ends were made flush by treatment with DNA polymerase I Klenow fragment and all four deoxyribonucleotides. A pair of complementary oligonucloeitdes of the following sequences were synthesized:

5'-TAGTAGGTAGCTAGCCA-3'
3'-ATCATCCATCGATCGGTCTAG-5'

When annealed to one another these olignucleotides encode translational terminators in all three reading frames. The annealed olignucleotide pair is flush-ended at one end and provides a four nucleotide single-stranded region capable of ligation to Bg1II digested DNA at the other end. The oligonucleotides were annealed to one another and ligated to pMON294 DNA which had been digested with BC1I and treated as described above. The ligated DNAs were digested with Bg1II, and a Bg1II fragment of approximately 1.9 kb containing the desired *B.t.* toxin coding sequence was isolated. The fragment extends from the Bg1II site just upstream of the translational initiation codon to the Bg1II site created by the oligonucleotide pair. The Bg1II fragment was ligated with Bg1II digested pMON316. A clone (pMON9713) was identified in which the translational initiator for the toxin gene was adjacent to the 35S promoter by digestion with HindIII.

EXAMPLES 6-8

CHIMERIC B.t. TOXIN GENES FOR PLANT TRANSFORMATION USING MAS PROMOTER

Three chimeric *B.t.* toxin genes were constructed in which the mannopine synthase (MAS) promoter from plasmid pTiA6, an octopine-type plasmid from *Agrobacterium tumefaciens* was used in combination with the *B.t.* toxin genes contained in previously described constructs pMON9711, pMON9712 and pMON9713.

The MAS promoter was isolated from pTiA6 as a 1.5 kb EcoRI-ClaI fragment. This DNA fragment extends from the ClaI site at nucleotide 20,138 to the EcoRI site at 21,631 in the sequence of Barker et al. (1983). Referring to Figure 9, the EcoRI-ClaI fragment was ligated with the binary vector pMON505 (Horsch et al. 1986) which had been previously digested with EcoRI and ClaI. The resulting plasmid was designated pMON706. A fragment containing the NOS 3' end was inserted downstream of the MAS promoter to obtain a MAS-NOS 3' expression cassette vector. The NOS 3' fragment was excised from pMON530 as a 300 bp BglII-BamHI fragment and inserted into BglII-digested pMON706. The resulting plasmid was designated pMON707.

Plasmid pMON530 was constructed by cleavage of pMON200 with NdeI to remove a 900 bp NdeI fragment to create pMON503. Plasmid pMON503 was cleaved with HindIII and SmaI and mixed with plasmid pTJS75 (Schmidhauser and Helinski, 1985) that had also been cleaved with HindIII and SmaI. A plasmid that contained the 3.8 kb HindIII-SmaI fragment of pTJS75 joined to the 8 kb HindIII-SmaI fragment of pMON503 was isolated and designated pMON505. Next the CaMV35S-NOS3' cassette was transferred to pMON505 by cleavage of pMON316 with StuI and HindII and isola tion of the 2.5 kb StuI-HindIII fragment containing the NOS-NPTII'-NOS marker and the CaMV35S-NOS3' cassette. This was added to pMON505 DNA cleaved with StuI and HindIII. Following ligation and transformation a plasmid carrying the CaMV35S-NOS3' cassette in pMON505 was

isolated and designated pMON530.

Since some binary vectors have greatly reduced frequencies of transformation in tomato as compared to co-integrating vectors, McCormick et al. (1986), the MAS-NOS 3′ cassette was moved from pMON707 into the co-integrating vector pMON200, Fraley et al. (1985). Plasmid pMON200 was digested with StuI and HindIII and a 7.7 kb fragment isolated by agarose gel electrophoresis. Plasmid pMON707 was similarly digested with StuI and HindIII and a 3.5 kb StuI-HindIII fragment containing the MAS-NOS 3′ cassette was isolated by agarose gel electrophoresis and recovery on a DEAE membranes with subsequent elution with 1M NaCl. These two DNA fragments were ligated and the resulting plasmid was designated pMON9741. This plasmid contains the MAS-NOS 3′ cassette in the pMON200 co-integrating background. Chimeric *B.t.* toxin genes driven by the MAS promoter were prepared. Referring to Figure 10, the *B.t.* toxin gene inserts were excised as BglII fragments from pMON9711, pMON9712 and pMON9713. Each of these fragments were ligated with BglII-digested pMON9741. In each case a clone in which the 5′ end of the *B.t.* toxin gene was adjacent to the MAS promoter by digestion with EcoRI. These plasmids were designated pMON9755, pMON9756 and pMON9757 and contained the *B.t.* toxin coding sequence from pMON9755, pMON9756 and pMON9713, respectively.

Plasmids pMON9755, pMON9756 and pMON9757 can be used to transform tomato cells as described above. Tomato plants regenerated therefrom will exhibit useful toxicity toward susceptible Lepidopteran larvae.

EXAMPLE 9

EXPRESSION OF CHIMERIC *B.t.* GENES IN TOMATO PLANTS

Introduction of Intermediate Vectors into *Agrobacterium*

Intermediate vectors containing truncated or full length toxin genes (pMON297, pMON8053, pMON9711, pMON9712 and pMON9713) were introduced into *Agrobacterium tumefaciens* strain GV311SE which contains the disarmed Ti plasmid pTiB6SE described by Fraley et al. (1985). *Agrobacterium tumefaciens* strains containing co-integrates between pTiB6SE and these intermediate vectors were selected as described.

Transformation and Regeneration of Tomato

Transformation and regeneration of transformed tomato utilizing the *Agrobacterium tumefaciens* strains described above was performed as described by McCormick et al. (1986).

Insect Feeding Assays of Transformed Tomato Plants

Toxicity of transformed tomato and tobacco plants containing chimeric *B.t.* toxin genes was determined by feeding assays with test insects. In some experiments leaves were removed from plants and placed either singly or in groups of up to five leaves in sterile 82 mm Petri dishes containing moistened sterile filter paper. Five to ten insect larvae were applied to the leaves and allowed to feed for three to seven days at which time the larvae were scored for mortality and relative size. Alternatively, larvae were applied to the leaves of whole tomato plants growing in four inch pots which were enclosed in plastic boxes. Ripe tomato fruit were assayed for toxicity by applying insect larvae directly to the intact fruit. All assays were performed at room temperature.

Manduca sexta larvae used in feeding assays were obtained as eggs from Carolina Biological Supply Co., hatched at room temperature and applied to leaves or plants immediately after hatching.

pMON9711 in Tomato

Two tomato plants transformed using plant transformation vector pMON9711 were isolated and designated #337 and #344.

Three *Manduca sexta* feeding assays were done with leaves from these two plants. The results are summarized in Table II.

Table II

Toxicity of *B.t.* Toxin Producing Transgenic Tomato Plants to *Manduca Sexta* (Hornworm) Neonates

Plant	Chimeric Gene	Nopaline Assay	Mortality (%) #1 4 dys	#2 4 dys	#2 7 dys	#3 3 dys
337	B.t.(9711)	+	87	40	67	100
344	B.t.(9711)	+	87	82	100	100
T30	none	-	22	0	0	0

Significant mortality of *Manduca sexta* was observed after feeding on these two plants compared to non-transformed tomato plants or transgenic plants containing no *B.t.* toxin gene.

Two *Heliothis virescens* feeding assays were done with leaves from plants #337 and #344. The results of these assays are shown in Table III.

Table III

Toxicity of *B.t.* Toxin Producing Transgenic Tomato Plants to *Heliothis virescens* (Budworm) Larvae

Plant	Chimeric Gene	Nopaline Assay	Mortality (%) Test #1 (4 days)	Test #2 (3 days)
337	B.t.(9711)	+	100	100
344	B.t.(9711)	+	2.5	0
T30	None	-	0	0

Significant mortality of *Heliothis virescens* was observed after feeding on plant #337 but not on plant #344. RNA was prepared from tomato callus tissue which had been transformed with pMON9711 and then selected for kanamycin resistance. RNA was also prepared from plants #337 and #344. In both cases the RNA was enriched for poly(A) containing RNA by oligo (dT) cellulose chromatography. The RNAs were subjected to agarose gel electrophoresis in the presence of formaldehyde and blotted to Genescreen® membranes. The membranes were hybridized with a ^{32}P labeled DNA fragment containing the *B.t.* toxin gene. Specific hybridization to the *B.t.* toxin gene was detected in RNA prepared from pMON9711 transformed tomato callus and plants #337 and #344. The *B.t.*-specific RNA was several-fold more abundant in plant #337 than in plant #344. It is believed that this difference is due to a position affect upon insertion of the toxin gene into the genome of the plant.

A *Heliothis virescens* feeding assay was performed on a tomato fruit from plant #337 with a non-transformed fruit as control. Nine larvae (six day old) were applied to each fruit and allowed to feed for four days. At the end of the test period there was a significant difference in larval weight between the two treatments. Larvae feeding on fruit from #337 had an average weight of 0.091 g (range = 0.061 g to 0.1445 g) while larvae feeding on the control fruit had an average weight of 0.1256 g (range = 0.093 g to 0.190 g).

A *Heliothis zea* feeding assay was done with leaves from plant #337. After five days mortality of the larvae was significantly higher on plant #337 than on a control plant. Fourteen of twenty larvae applied to leaves of #337 had died compared to only eight of twenty larvae on the control leaves.

F1 progeny plants from self-crosses of plants #337 and #344 were isolated. The progeny were tested for toxicity in the *Manduca sexta* feeding assay four to six weeks after planting. The progeny were also tested for nopaline production. For both #337 and #344 the progeny segregated both nopaline production and high level toxicity to *Manduca sexta*. Many progeny were as toxic to the larvae as the parental transformed plants.

For progeny of both plants there was a good correlation between high level toxicity and nopaline production.

pMON9712 in Tomato

One tomato plant transformed using plant transformation vector pMON9712 was isolated as described above. Eleven F1 progeny from a self cross of this plant were recovered and assayed for toxicity to *Manduca sexta* neonatal larvae in two separate tests. The insect feeding assays were performed on whole plants as previously described.

Nine of the eleven progeny tested expressed nopaline synthase indicating the presence of the transforming DNA. Fewer than half of the larvae survived on eight of the nine nopaline positive plants. Fewer than twenty percent of the larvae survived on two of the nine nopaline positive plants. The survival rate for larvae on nontransformed plants was about ninety percent. These results indicate a significant level of toxicity in tomato plants transformed with pMON9712 as compared to nontransformed plants.

pMON9713 in Tomato

One tomato plant transformed using plant transformation vector pMON9713 was isolated as described above. This plant was assayed for toxicity to *Manduca sexta* neonatal larvae as described above. In the feeding assay all ten larvae applied to this plant were killed. Nine of ten larvae applied to a nontransformed tomato plant survived.

This result indicates a significant level of toxicity in tomato plants transformed with pMON9713 as compared to nontransformed plants.

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Claims

1. A method of producing genetically transformed tomato plants which exhibit toxicity toward Lepidopteran larvae which comprises:
   (a) inserting into the genome of a tomato cell a chimeric gene which comprises
      (i) a promoter which functions in plants to cause the production of a mRNA transcript;
      (ii) a coding sequence that causes the production of mRNA encoding a toxin protein of *Bacillus thuringiensis*; and
      (iii) a 3′ non-translated region which functions in tomato to cause the addition of polyadenylate nucleotides to the 3′ end of the mRNA;
   (b) selecting transformed tomato cells; and
   (c) regenerating from the transformed tomato cells genetically transformed tomato plants which exhibit toxicity toward Lepidopteran larvae.
2. A method of Claim 1 in which the promoter is selected from the group consisting of the CaMV35S and MAS promoters.
3. A method of Claim 2 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.
4. A method of claim 2 in which the coding sequence encodes the protein encoded by DNA sequence 1-2170 of Figure 1.
5. A method of Claim 2 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.
6. A method of Claim 1 in which the promoter is the CaMV35S promoter.
7. A method of Claim 6 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.
8. A method of Claim 6 in which the coding sequence encodes the protein encoded by DNA sequence 1-2170 of Figure 1.
9. A method of Claim 6 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.
10. A transformed tomato cell exhibiting toxicity toward Lepidopteran larvae containing a chimeric gene comprising in sequence:
   (a) a promoter which functions in plants to cause the production of a mRNA transcript;
   (b) a coding sequence that causes the production of mRNA encoding a toxin protein of *Bacillus thuringiensis*; and
   (c) a 3′ non-translated region which functions in tomato to cause the addition of polyadenylate nucleotides to the 3′ end of the mRNA.
11. A transformed tomato cell of Claim 10 in which the promoter is selected from the group consisting of CaMV35S and MAS promoters.

12. A cell of Claim 11 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.

13. A cell of Claim 11 in which the coding sequence encodes the protein encoded by DNA sequences 1-2170 of Figure 1.

14. A cell of Claim 11 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.

15. A cell of Claim 10 in which the promoter is the CaMV35S promoter.

16. A cell of Claim 15 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.

17. A cell of Claim 15 in which the coding sequence encodes the protein encoded by DNA sequence 1-2170 of Figure 1.

18. A cell of Claim 15 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.

19. A differentiated tomato plant which exhibit toxicity toward Lepidopteran larvae comprising transformed tomato cells of Claim 10.

20. A differentiated plant of Claim 19 in which the promoter is selected from the group consisting of CaMV35S and MAS promoters.

21. A differentiated plant of Claim 20 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.

22. A differentiated plant of Claim 20 in which the coding sequence encodes the protein encoded by DNA sequence 1-2170 of Figure 1.

23. A differentiated plant of Claim 20 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.

24. A differentiated tomato plant of Claim 20 in which the promoter is the CaMV35S promoter.

25. A differentiated plant of Claim 24 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.

26. A differentiated plant of Claim 24 in which the coding sequence encodes the protein encoded by DNA sequence 1-2170 of Figure 1.

27. A differentiated plant of Claim 24 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.

28. A chimeric plant gene which comprises:

(i) a promoter which function in plants to cause the production of a mRNA transcript;

(ii) a coding sequence that causes the production of mRNA encoding a toxin protein of *Bacillus thuringiensis*; and

(iii) a 3′ non-translated region which functions in plants to cause the addition of polyadenylate nucleotides to the 3′ end of the mRNA.

29. A chimeric plant gene of Claim 28 in which the promoter is selected from the group consisting of CaMV35S and MAS promoter.

30. A chimeric plant gene of Claim 29 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.

31. A chimeric plant gene of Claim 29 in which the coding sequence encodes the protein encoded by the DNA sequence 1-2170 of Figure 1.

32. A chimeric plant gene of Claim 29 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.

33. A chimeric plant gene of Claim 28 in which the promoter is the CaMV35S promoter.

34. A chimeric plant gene of Claim 33 in which the coding sequence encodes the protein encoded by DNA sequence 1-3471 of Figure 1.

35. A chimeric plant gene of Claim 33 in which the coding sequence encodes the protein encoded by the DNA sequence 1-2170 of Figure 1.

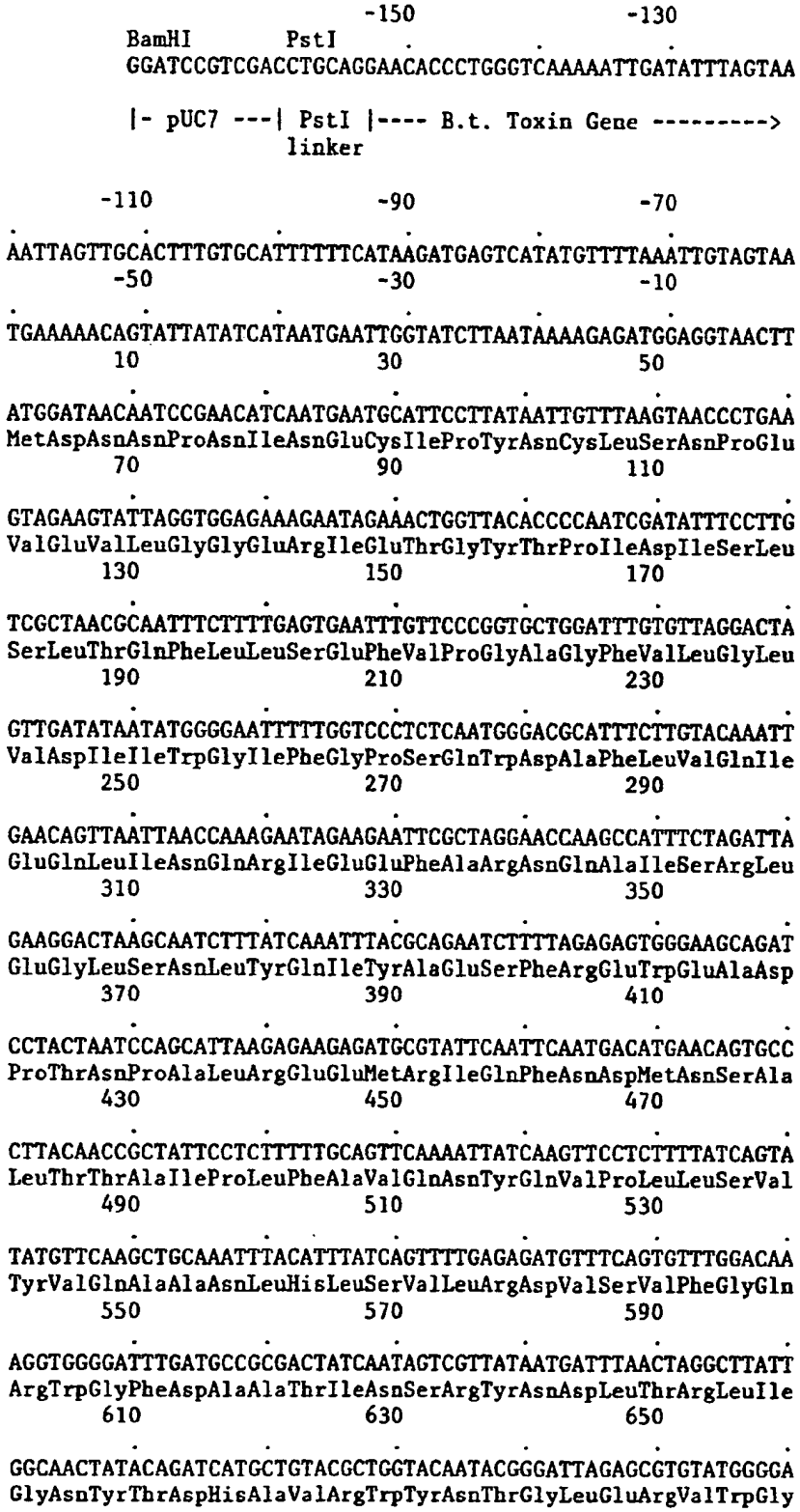
36. A chimeric plant gene of Claim 33 in which the coding sequence encodes the protein encoded by DNA sequence 1-1935 of Figure 1.

37. The method of Claim 6 in which the protein toxin is from *Bacillus thuringiensis* subspecies *kurstaki* and the transformed plant exhibits toxicity toward Lepidopterans of the genus *Manduca* and *Heliothis* and *Spodotera exigua.*

38. The cell of Claim 15 in which the protein toxin is from *Bacillus thuringiensis* subspecies *kurstaki* and the transformed plant exhibits toxicity toward Lepidopterans of the genus *Manduca* and *Heliothis* and *Spodotera exigua.*

39. A differentiated tomato plant of Claim 20 in which the promoter is the CaMV35S promoter, the protein toxin is from *Bacillus thuringiensis* subspecies *kurstaki* and the transformed plant exhibits toxicity toward Lepidopterans of the genus *Manduca* and *Heliothis* and *Spodotera exigua.*

40. A chimeric plant gene of Claim 33 in which the protein toxin is from *Bacillus thuringiensis* subspecies *kurstaki.*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|- pUC7 ---| PstI |---- B.t. Toxin Gene --------->
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-90
-70
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-30
-10
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90
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Fig 1(a)

```
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       790                 810                 830
TCCCAATTAACAAGAGAAATTTATACAAACCCAGTATTAGAAAATTTTGATGGTAGTTTT
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      1150                1170                1190
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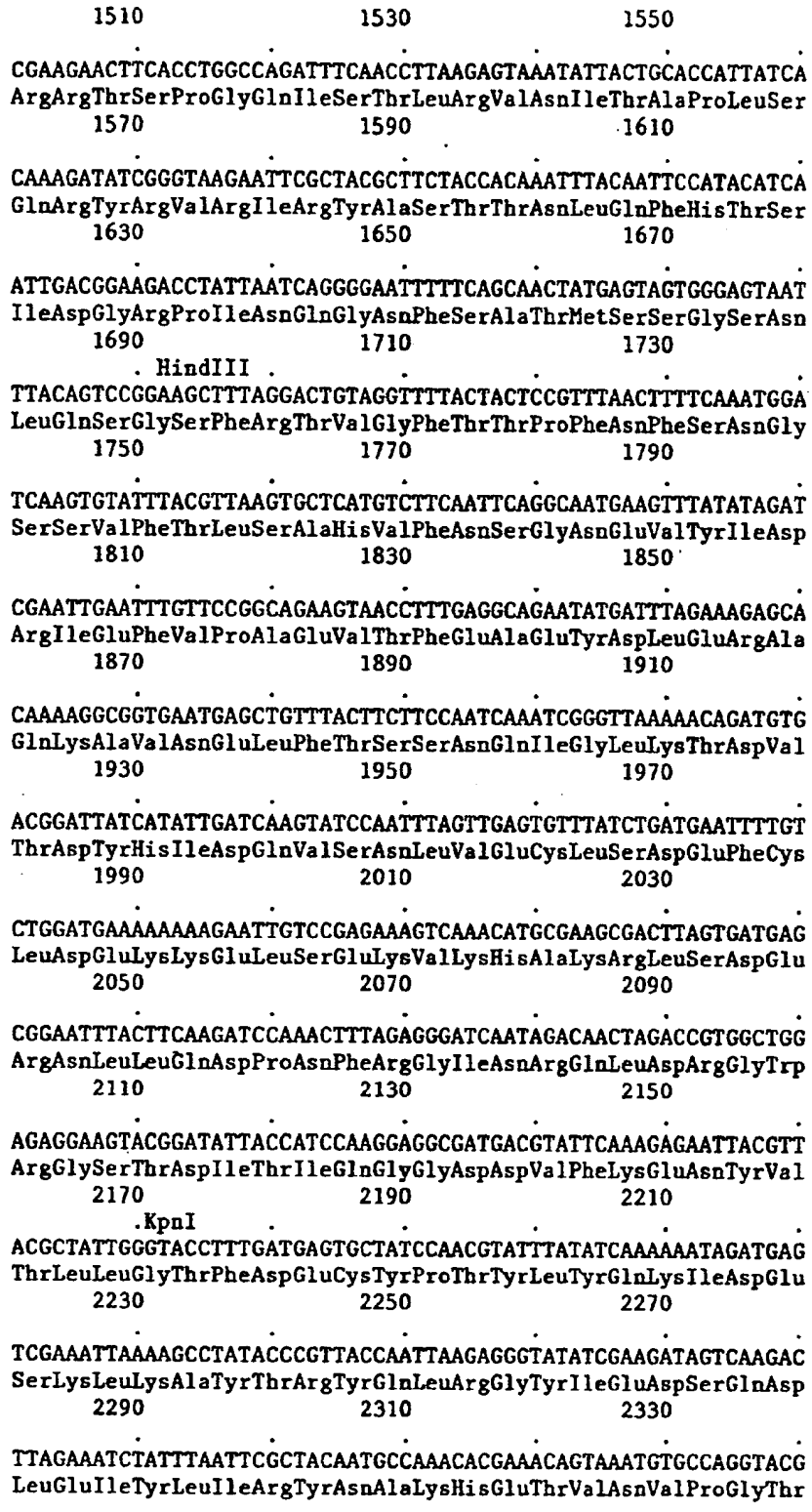
Fig 1(b)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1650
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1730
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1970
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2010
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2310
2330
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Fig 1(c)

```
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       .                   .                   .
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       .                   .                   .
CATTTCTCCTTGGACATTGATGTTGGATGTACAGACTTAAATGAGGACTTAGGTGTATGG
HisPheSerLeuAspIleAspValGlyCysThrAspLeuAsnGluAspLeuGlyValTrp
      2470                2490                2510
       .                   .                   .
GTGATATTCAAGATTAAGACGCAAGATGGCCATGAAAGACTAGGAAATCTAGAATTTCTC
ValIlePheLysIleLysThrGlnAspGlyHisGluArgLeuGlyAsnLeuGluPheLeu
      2530                2550                2570
       .                   .                   .
GAAGGAAGAGCACCATTAGTAGGAGAAGCACTAGCTCGTGTGAAAAGAGCGGAGAAAAAA
GluGlyArgAlaProLeuValGlyGluAlaLeuAlaArgValLysArgAlaGluLysLys
      2590                2610                2630
       .                   .                   .
TGGAGAGACAAACGTGAAAAATTGGAATGGGAAACAAATATTGTTTATAAAGAGGCAAAA
TrpArgAspLysArgGluLysLeuGluTrpGluThrAsnIleValTyrLysGluAlaLys
      2650                2670                2690
       .                   .                   .
GAATCTGTAGATGCTTTATTTGTAAACTCTCAATATGATAGATTACAAGCGGATACCAAC
GluSerValAspAlaLeuPheValAsnSerGlnTyrAspArgLeuGlnAlaAspThrAsn
      2710                2730                2750
 NruI  .                   .                   .  HindIII    .
ATCGCGATGATTCATGCGGCAGATAAACGCGTTCATAGCATTCGAGAAGCTTATCTGCCT
IleAlaMetIleHisAlaAlaAspLysArgValHisSerIleArgGluAlaTyrLeuPro
      2770                2790                2810
       .                   .                   .
GAGCTGTCTGTGATTCCGGGTGTCAATGCGGCTATTTTTGAAGAATTAGAAGGGCGTATT
GluLeuSerValIleProGlyValAsnAlaAlaIlePheGluGluLeuGluGlyArgIle
      2830                2850                2870
       .                   .                   .
TTCACTGCATTCTCCCTATATGATGCGAGAAATGTCATTAAAAATGGTGATTTTAATAAT
PheThrAlaPheSerLeuTyrAspAlaArgAsnValIleLysAsnGlyAspPheAsnAsn
      2890                2910                2930
       .                   .                   .
GGCTTATCCTGCTGGAACGTGAAAGGGCATGTAGATGTAGAAGAACAAAACAACCACCGT
GlyLeuSerCysTrpAsnValLysGlyHisValAspValGluGluGlnAsnAsnHisArg
      2950                2970                2990
       .                   .                   .
TCGGTCCTTGTTGTTCCGGAATGGGAAGCAGAAGTGTCACAAGAAGTTCGTGTCTGTCCG
SerValLeuValValProGluTrpGluAlaGluValSerGlnGluValArgValCysPro
      3010                3030                3050
       .                   .                   .
GGTCGTGGCTATATCCTTCGTGTCACAGCGTACAAGGAGGGATATGGAGAAGGTTGCGTA
GlyArgGlyTyrIleLeuArgValThrAlaTyrLysGluGlyTyrGlyGluGlyCysVal
      3070                3090                3110
       .                   .                   .
ACCATTCATGAGATCGAGAACAATACAGACGAACTGAAGTTTAGCAACTGTGTAGAAGAG
ThrIleHisGluIleGluAsnAsnThrAspGluLeuLysPheSerAsnCysValGluGlu
      3130                3150                3170
       .                   .                   .
GAAGTATATCCAAACAACACGGTAACGTGTAATGATTATACTGCGACTCAAGAAGAATAT
GluValTyrProAsnAsnThrValThrCysAsnAspTyrThrAlaThrGlnGluGluTyr
```

Fig 1(d)

```
      3190                3210                3230
       .                   .                   .                   .
GAGGGTACGTACACTTCTCGTAATCGAGGATATGACGGAGCCTATGAAAGCAATTCTTCT
GluGlyThrTyrThrSerArgAsnArgGlyTyrAspGlyAlaTyrGluSerAsnSerSer
      3250                3270                3290
       .                   .                   .                   .
GTACCAGCTGATTATGCATCAGCCTATGAAGAAAAAGCATATACAGATGGACGAAGAGAC
ValProAlaAspTyrAlaSerAlaTyrGluGluLysAlaTyrThrAspGlyArgArgAsp
      3310                3330                3350
       .                   .                   .                   .
AATCCTTGTGAATCTAACAGAGGATATGGGGATTACACACCACTACCAGCTGGCTATGTG
AsnProCysGluSerAsnArgGlyTyrGlyAspTyrThrProLeuProAlaGlyTyrVal
      3370                3390                3410
       .  ScaI             .                   .                   .
ACAAAAGAATTAGAGTACTTCCCAGAAACCGATAAGGTATGGATTGAGATCGGAGAAACG
ThrLysGluLeuGluTyrPheProGluThrAspLysValTrpIleGluIleGlyGluThr
      3430                3450                3470
       .                   .                   .                   .
GAAGGAACATTCATCGTGGACAGCGTGGAATTACTTCTTATGGAGGAATAATATATGCTT
GluGlyThrPheIleValAspSerValGluLeuLeuMetGluGluEnd
      3490                3510                3530
       .                   .                   .                   .
TAAAATGTAAGGTGTGCAAATAAAGAATGATTACTGACTTGTATTGACAGATAAATAAGG
      3550                3570                3590
       .                   .                   .                   .
AAATTTTTATATGAATAAAAAACGGGCATCACTCTTAAAAGAATGATGTCCGTTTTTTGT
      3610                3630                3650
       .                   .                   .                   .  KpnI
ATGATTTAACGAGTGATATTTAAATGTTTTTTTGCGAAGGCTTTACTTAACGGGGTACC
```

Fig 1 (e)

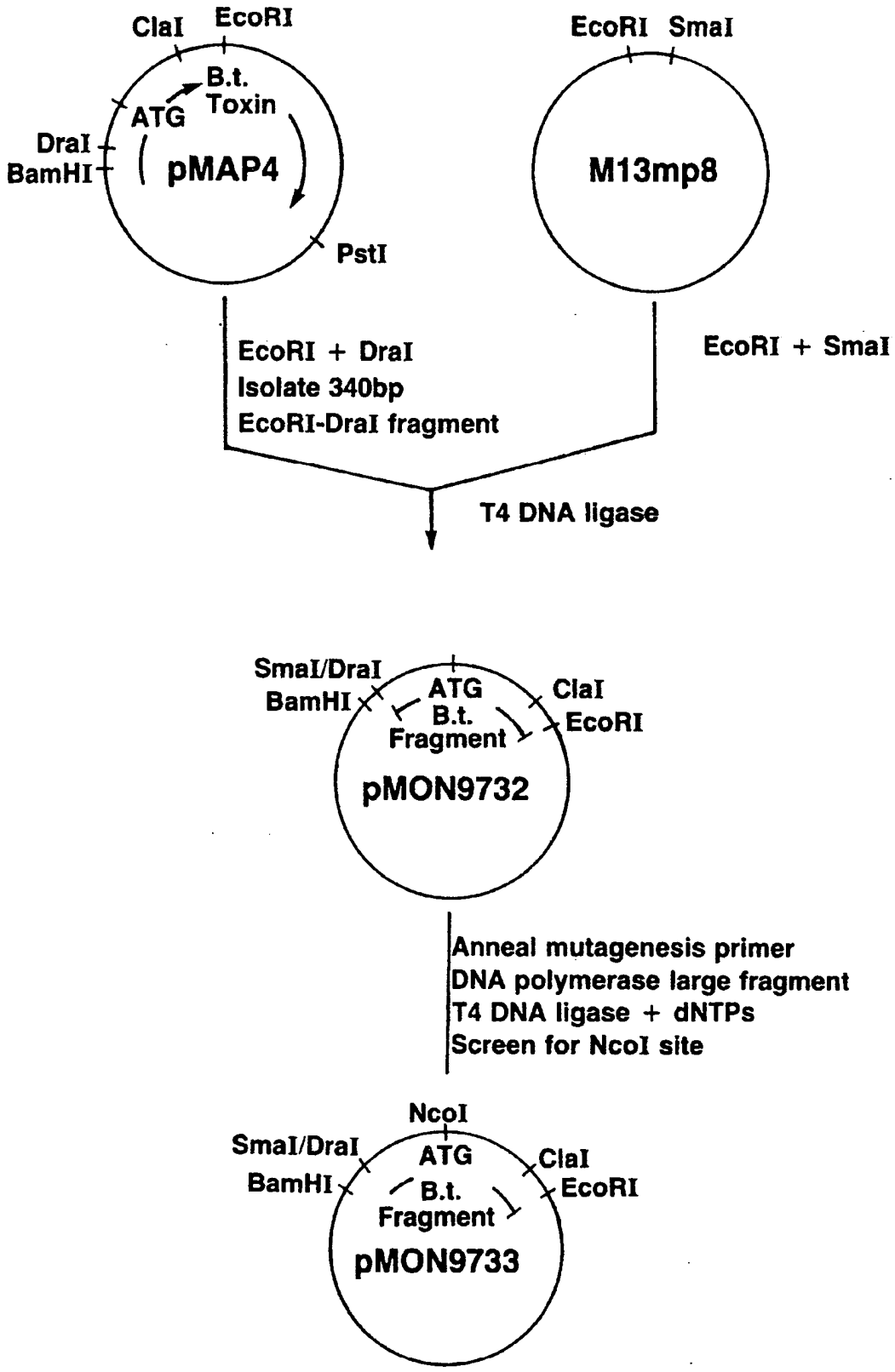
ClaI
EcoRI
B.t.
Toxin
ATG
DraI
BamHI
pMAP4
PstI
EcoRI
SmaI
M13mp8
EcoRI + DraI
Isolate 340bp
EcoRI-DraI fragment
EcoRI + SmaI
T4 DNA ligase
SmaI/DraI
BamHI
ATG
B.t.
Fragment
ClaI
EcoRI
pMON9732
Anneal mutagenesis primer
DNA polymerase large fragment
T4 DNA ligase + dNTPs
Screen for NcoI site
NcoI
SmaI/DraI
BamHI
ATG
B.t.
Fragment
ClaI
EcoRI
pMON9733

FIG 2

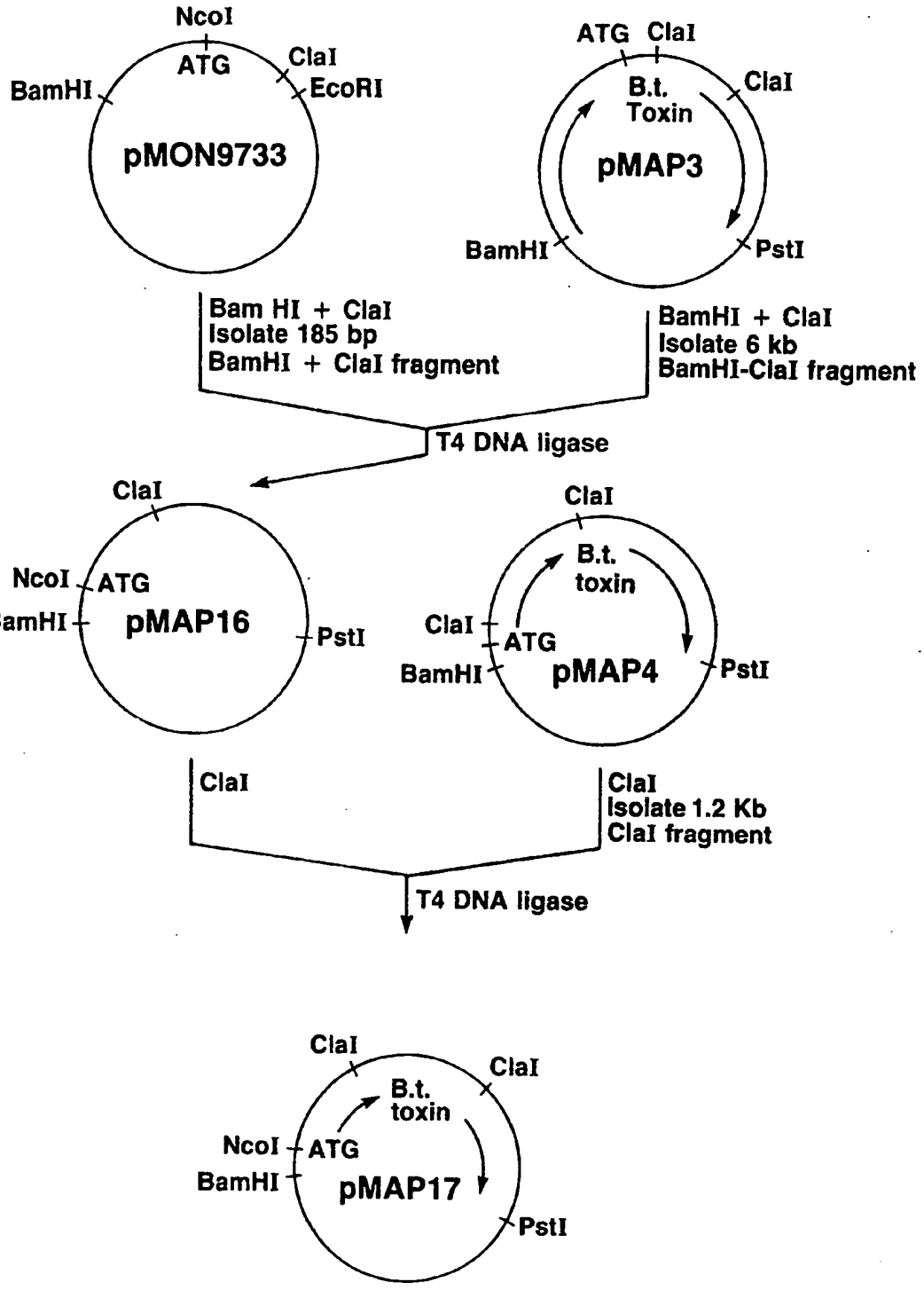
NcoI
ATG
ClaI
EcoRI
BamHI
pMON9733
ATG ClaI
B.t.
Toxin
ClaI
pMAP3
BamHI
PstI
Bam HI + ClaI
Isolate 185 bp
BamHI + ClaI fragment
BamHI + ClaI
Isolate 6 kb
BamHI-ClaI fragment
T4 DNA ligase
ClaI
NcoI
ATG
BamHI
pMAP16
PstI
ClaI
B.t.
toxin
ClaI
ATG
BamHI
pMAP4
PstI
ClaI
ClaI
Isolate 1.2 Kb
ClaI fragment
T4 DNA ligase
ClaI
ClaI
B.t.
toxin
NcoI
ATG
BamHI
pMAP17
PstI

FIG 3

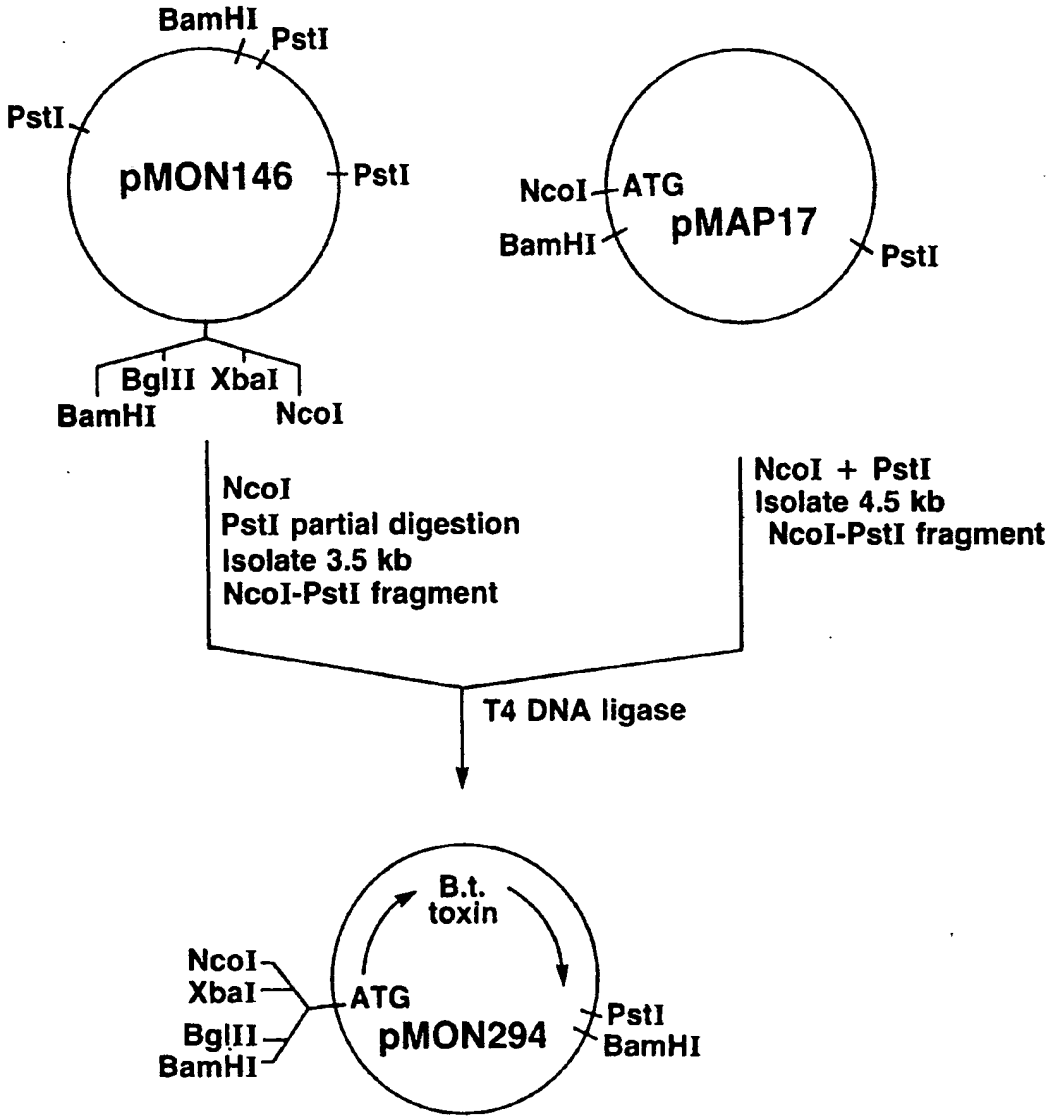
BamHI
PstI
PstI
pMON146
PstI
BglII XbaI
BamHI
NcoI
NcoI
ATG
BamHI
pMAP17
PstI
NcoI
PstI partial digestion
Isolate 3.5 kb
NcoI-PstI fragment
NcoI + PstI
Isolate 4.5 kb
NcoI-PstI fragment
T4 DNA ligase
B.t.
toxin
NcoI
XbaI
BglII
BamHI
ATG
pMON294
PstI
BamHI

FIG 4

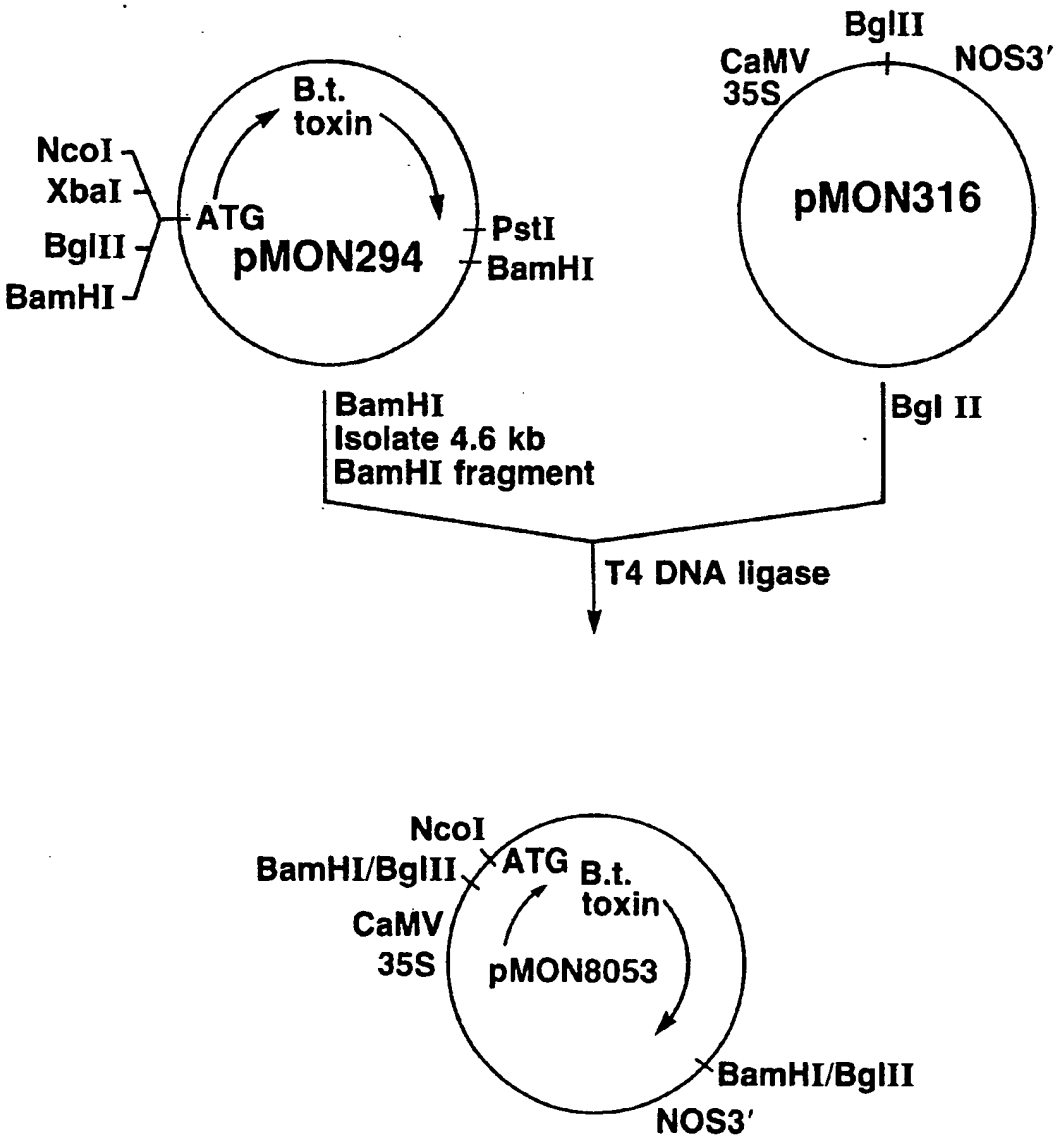
BglII
CaMV
35S
NOS3'
pMON316
B.t.
toxin
NcoI
XbaI
BglII
BamHI
ATG
pMON294
PstI
BamHI
BamHI
Isolate 4.6 kb
BamHI fragment
Bgl II
T4 DNA ligase
NcoI
BamHI/BglII
ATG
B.t.
toxin
CaMV
35S
pMON8053
BamHI/BglII
NOS3'

FIG 5

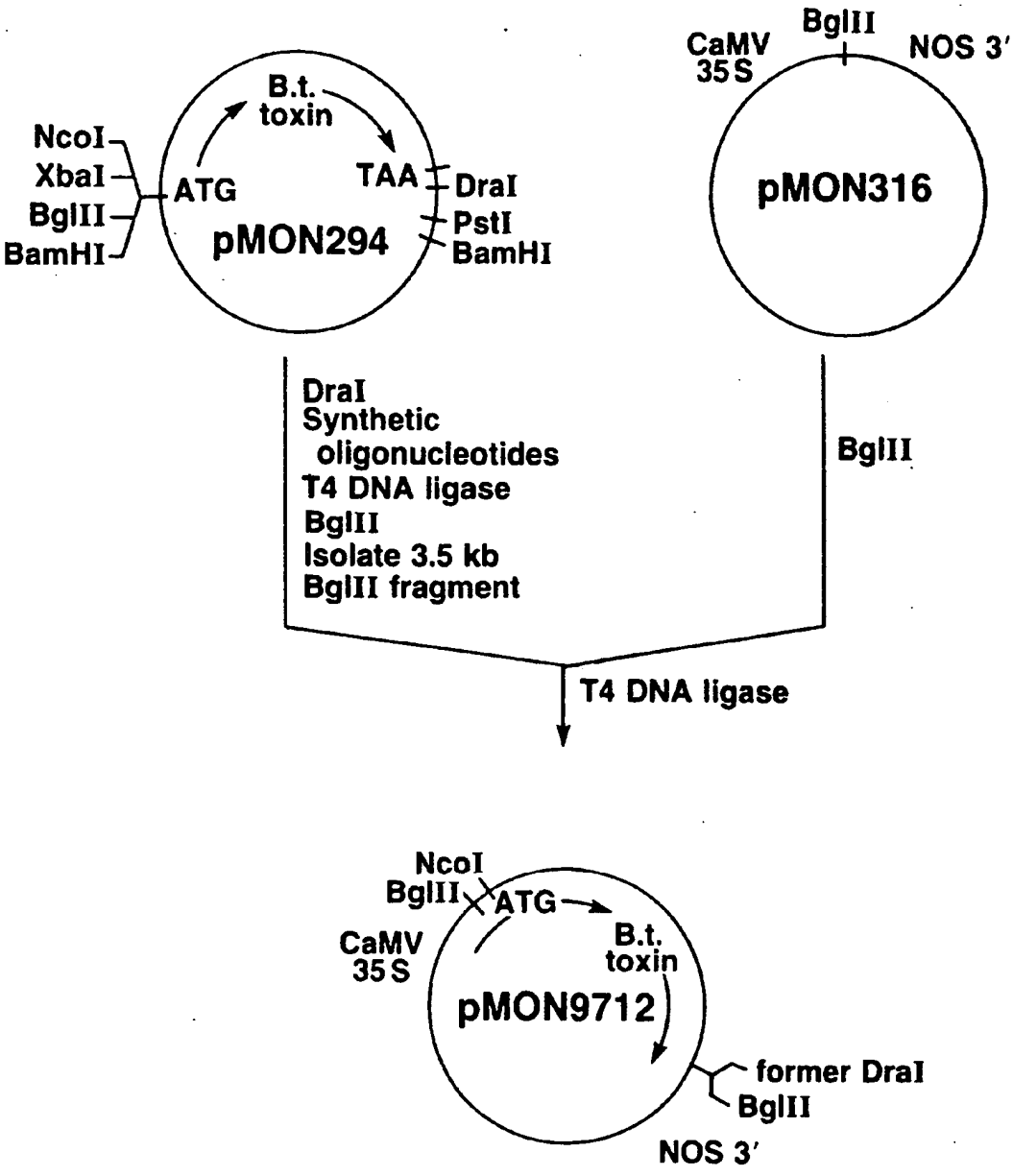
NcoI
XbaI
BglII
BamHI
ATG
B.t. toxin
TAA
DraI
PstI
BamHI
pMON294
CaMV 35S
BglII
NOS 3'
pMON316
DraI
Synthetic oligonucleotides
T4 DNA ligase
BglII
Isolate 3.5 kb BglII fragment
BglII
T4 DNA ligase
NcoI
BglII
ATG
CaMV 35S
B.t. toxin
pMON9712
former DraI
BglII
NOS 3'

FIG 6

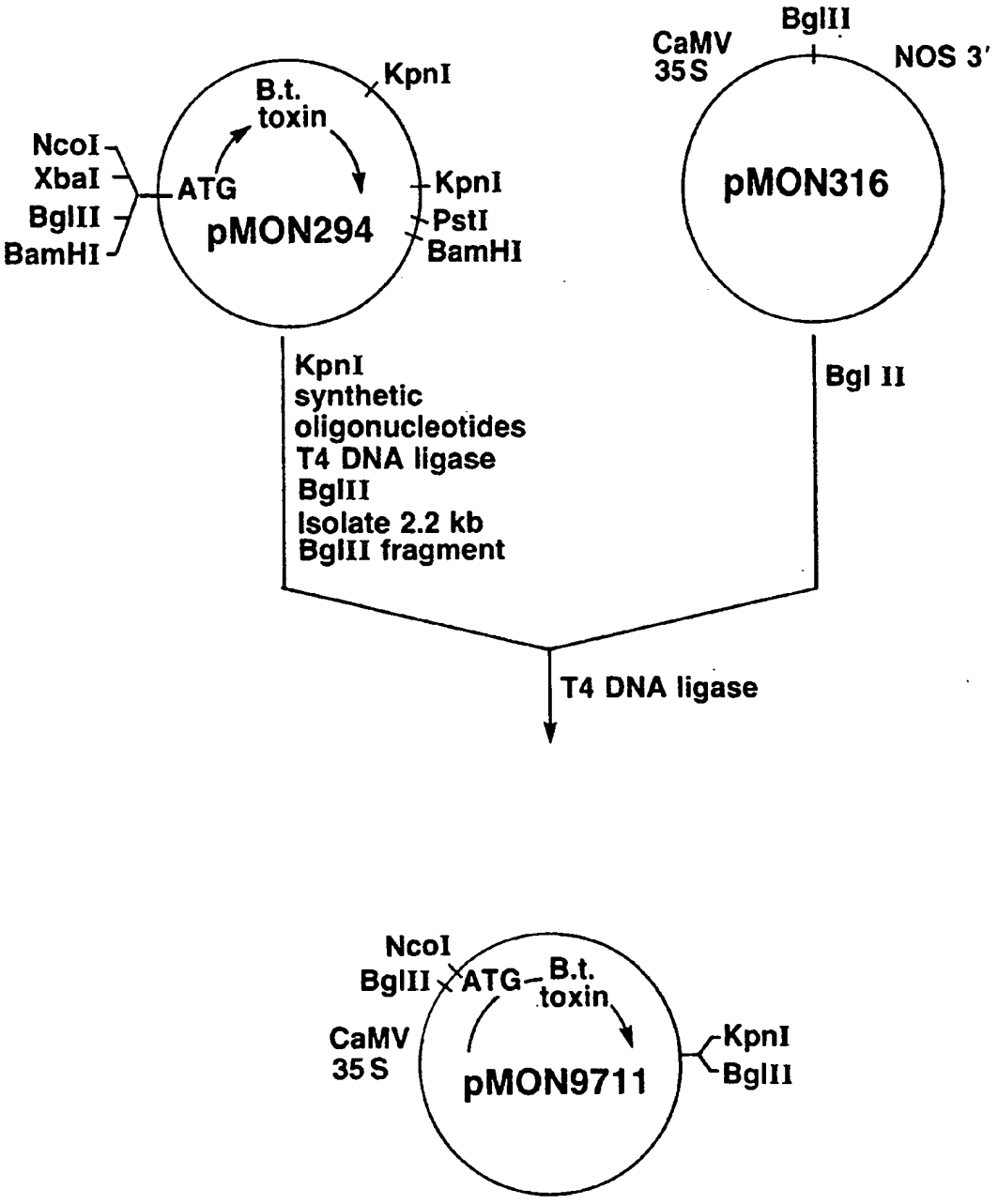
B.t.
toxin
KpnI
NcoI
XbaI
BglII
BamHI
ATG
pMON294
KpnI
PstI
BamHI
CaMV
35S
BglII
NOS 3'
pMON316
KpnI
synthetic
oligonucleotides
T4 DNA ligase
BglII
Isolate 2.2 kb
BglII fragment
Bgl II
T4 DNA ligase
NcoI
BglII
ATG
B.t.
toxin
CaMV
35S
pMON9711
KpnI
BglII

FIG 7

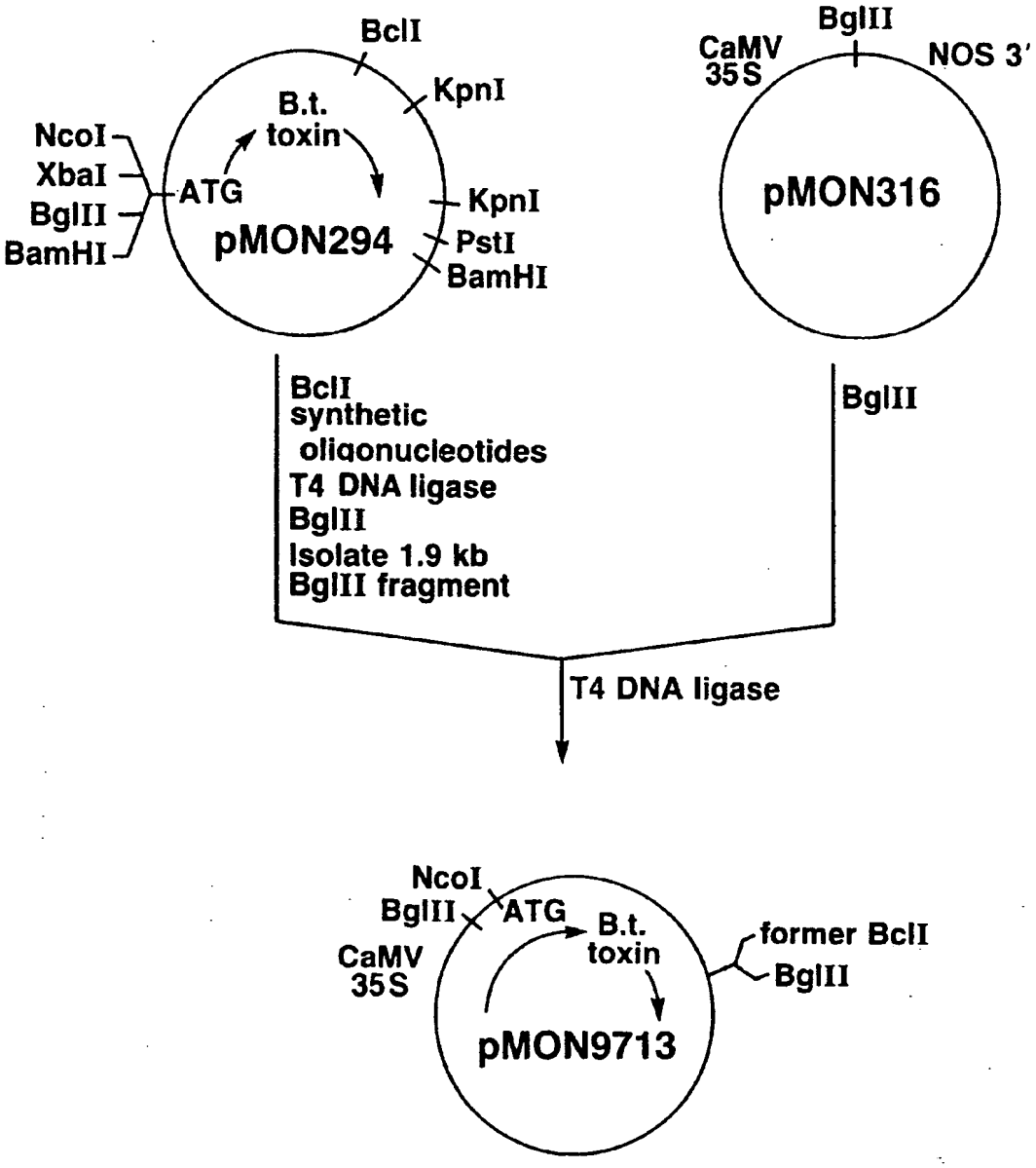
BclI
KpnI
B.t.
toxin
NcoI
XbaI
BglII
BamHI
ATG
KpnI
PstI
BamHI
pMON294
BglII
CaMV
35S
NOS 3'
pMON316
BclI
synthetic
oligonucleotides
T4 DNA ligase
BglII
Isolate 1.9 kb
BglII fragment
BglII
T4 DNA ligase
NcoI
BglII
ATG
B.t.
toxin
CaMV
35S
former BclI
BglII
pMON9713

FIG 8

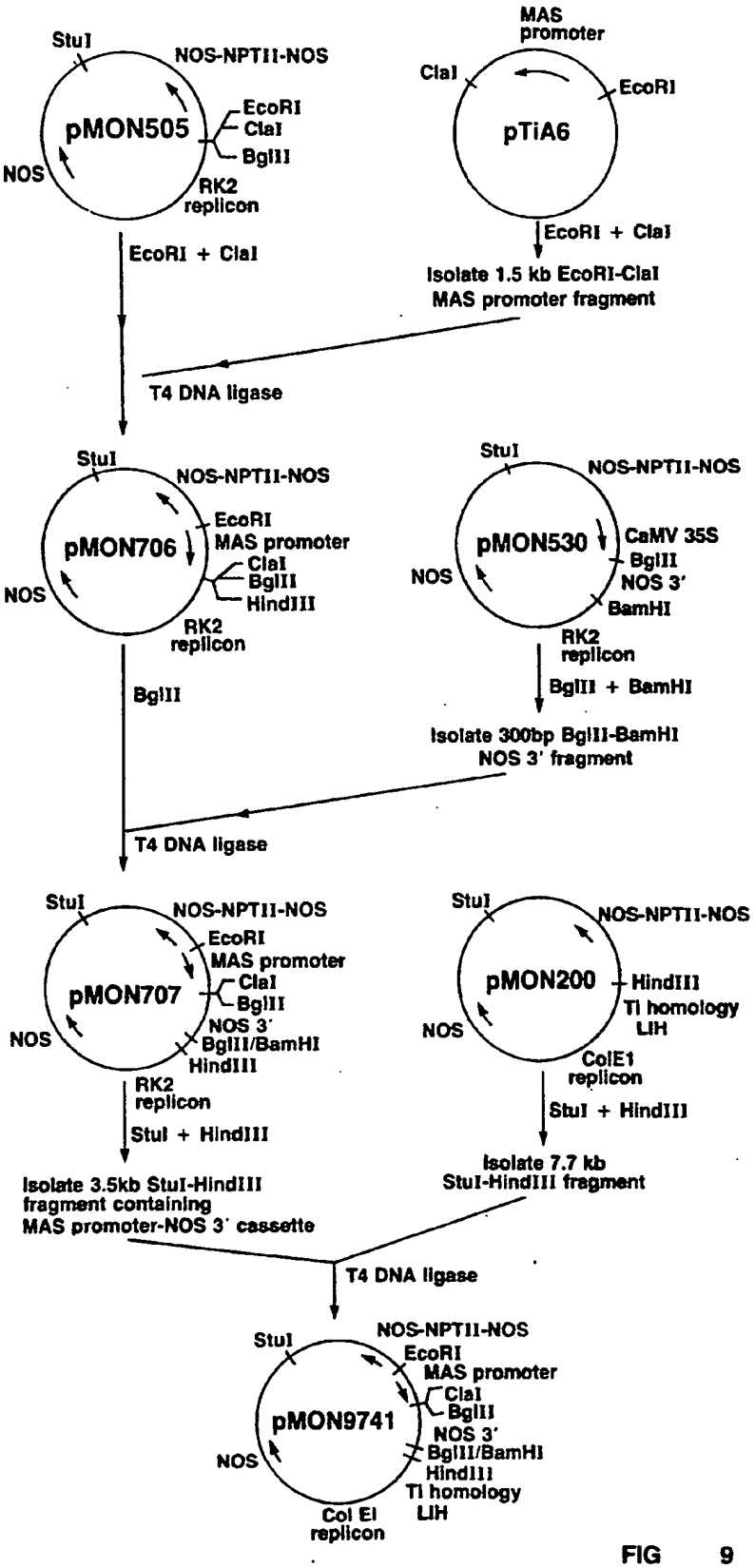
StuI
NOS-NPTII-NOS
pMON505
EcoRI
ClaI
BglII
NOS
RK2
replicon
EcoRI + ClaI
MAS
promoter
ClaI
EcoRI
pTiA6
EcoRI + ClaI
Isolate 1.5 kb EcoRI-ClaI
MAS promoter fragment
T4 DNA ligase
StuI
NOS-NPTII-NOS
pMON706
EcoRI
MAS promoter
ClaI
BglII
HindIII
NOS
RK2
replicon
BglII
StuI
NOS-NPTII-NOS
pMON530
CaMV 35S
BglII
NOS 3'
BamHI
NOS
RK2
replicon
BglII + BamHI
Isolate 300bp BglII-BamHI
NOS 3' fragment
T4 DNA ligase
StuI
NOS-NPTII-NOS
pMON707
EcoRI
MAS promoter
ClaI
BglII
NOS 3'
BglII/BamHI
HindIII
NOS
RK2
replicon
StuI + HindIII
Isolate 3.5kb StuI-HindIII
fragment containing
MAS promoter-NOS 3' cassette
StuI
NOS-NPTII-NOS
pMON200
HindIII
TI homology
LIH
NOS
ColE1
replicon
StuI + HindIII
Isolate 7.7 kb
StuI-HindIII fragment
T4 DNA ligase
StuI
NOS-NPTII-NOS
EcoRI
MAS promoter
ClaI
BglII
pMON9741
NOS 3'
BglII/BamHI
HindIII
TI homology
LIH
NOS
Col EI
replicon

FIG 9

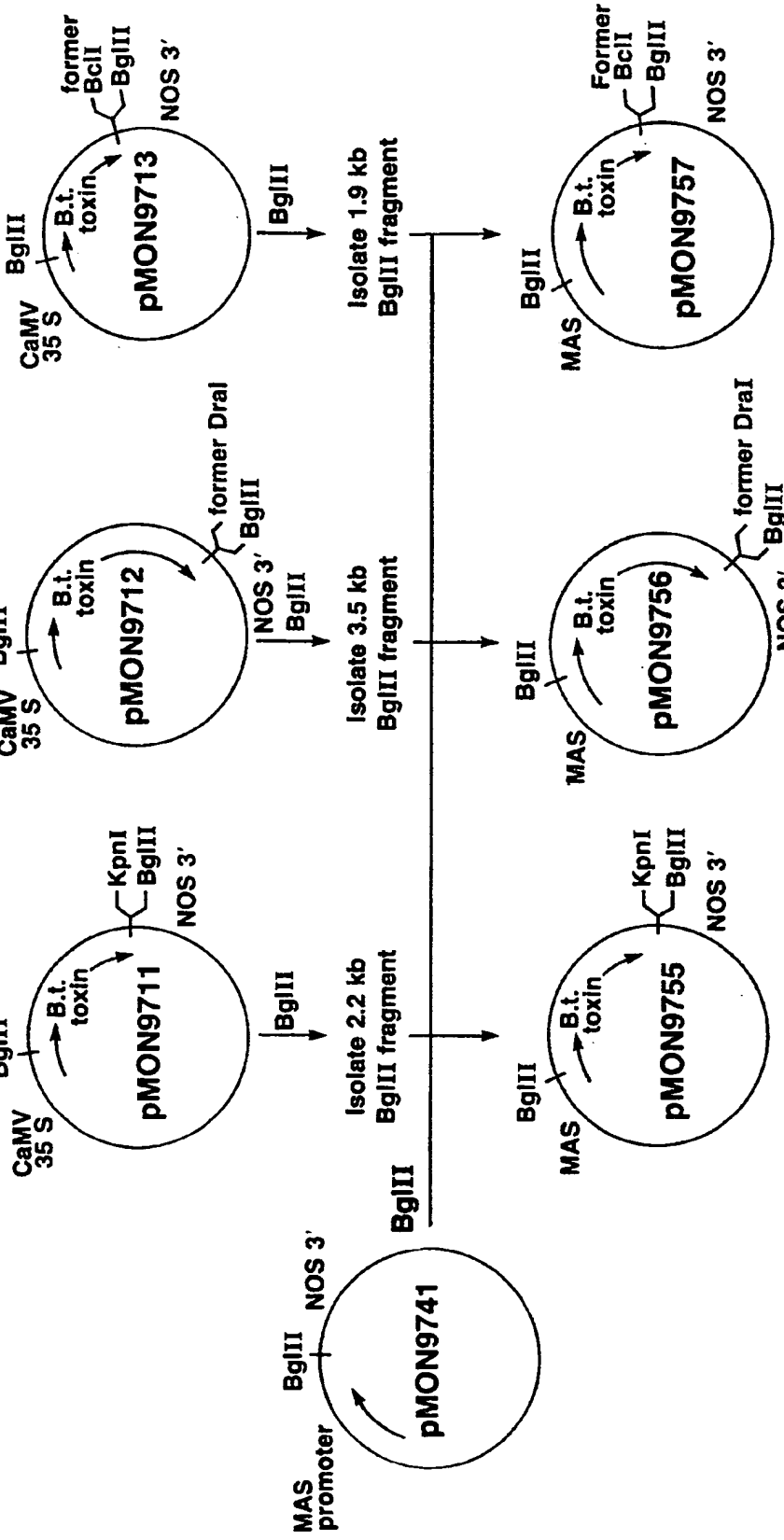
pMON9713
CaMV 35 S
BglII
B.t. toxin
former BclI
BglII
NOS 3'
BglII
Isolate 1.9 kb BglII fragment
pMON9757
MAS
BglII
B.t. toxin
Former BclI
BglII
NOS 3'
pMON9712
CaMV 35 S
BglII
B.t. toxin
former DraI
BglII
NOS 3'
BglII
Isolate 3.5 kb BglII fragment
pMON9756
MAS
BglII
B.t. toxin
former DraI
BglII
NOS 3'
pMON9711
CaMV 35 S
BglII
B.t. toxin
KpnI
BglII
NOS 3'
BglII
Isolate 2.2 kb BglII fragment
pMON9755
MAS
BglII
B.t. toxin
KpnI
BglII
NOS 3'
BglII
pMON9741
MAS promoter
BglII
NOS 3'

FIG 10

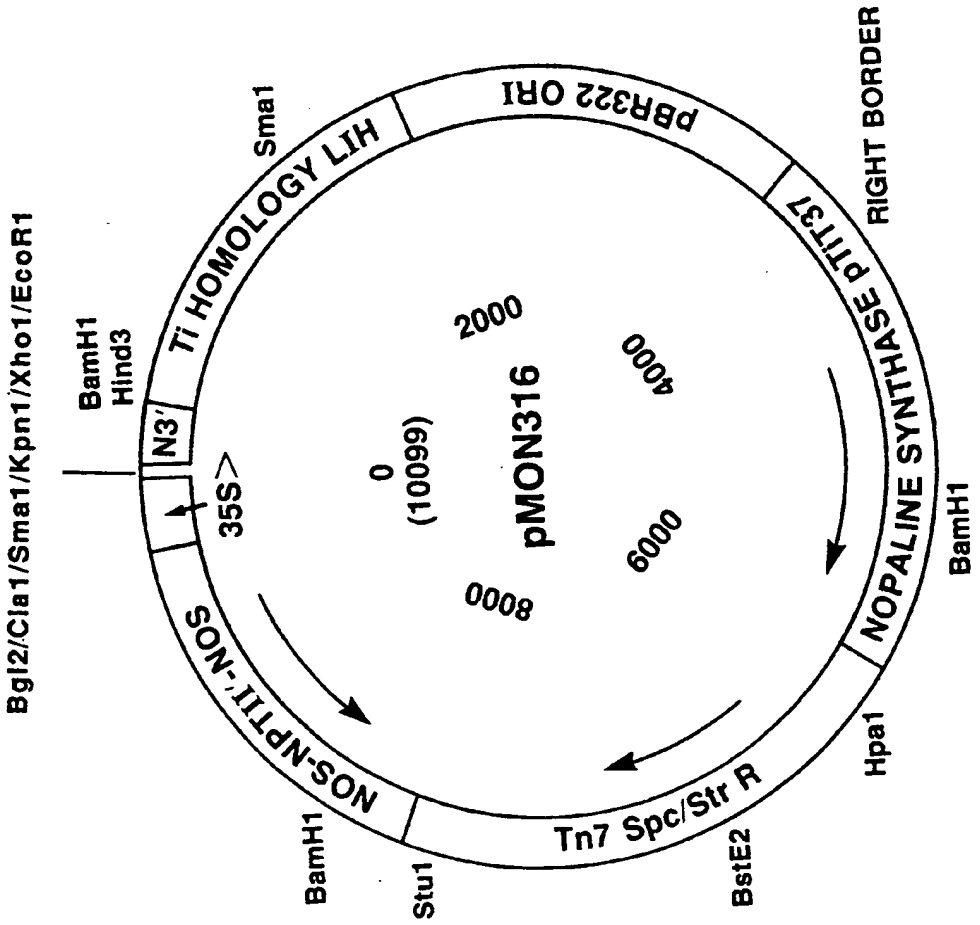
pMON316
0
(10099)
2000
4000
6000
8000
Bgl2/Cla1/Sma1/Kpn1/Xho1/EcoR1
BamH1
Hind3
N3'
Ti HOMOLOGY LIH
Sma1
pBR322 ORI
RIGHT BORDER
pTiT37
NOPALINE SYNTHASE
BamH1
Hpa1
BstE2
Tn7 Spc/Str R
Stu1
BamH1
NOS-NPTII'-NOS
35S>

FIG 11